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Cloning and heterologous expression of the extracellular alpha-galactosidase from *Aspergillus fumigatus* in *Aspergillus sojae* under the control of *gpd*A promoter

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ABSTRACT

Aspergillus fumigatus is highly pathogenic especially for immunocompromised people however it can efficiently produce many industrially important enzymes. The gene coding α-galactosidase enzyme (*agl*B) of *A. fumigatus* IMI 385708 has been cloned onto pAN52-4 fungal expression vector and expressed in a GRAS organism, *Aspergillus sojae* ATCC11906 under the control of constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) promoter. pAN52-4 fungal expression system allowed high level α-galactosidase production in media with simple sugar glucose as the sole carbon source and without a requirement for an inducer with a yield of 2.45 U/ml which is nearly 3-fold higher than the yield obtained from *A. fumigatus* grown in locust bean gum containing medium.

1. Introduction

α-Galactosidase (alpha-D-galactoside galactohydrolase; EC 3.2.1.22) is an exoglycosidase that catalyzes the hydrolysis of the terminal non-reducing α-1.6-linked α-galactose residues from oligosaccharides such as melibiose, raffinose, and stachyose and from polymeric galactomannans and galactolipids. Some α-galactosidases can also catalyze transgalactosylation reactions, especially at high substrate concentration [1]. *A. fumigatus* α-galactosidase has also been shown to catalyze efficient transgalactosylation reaction to a variety of mono- and oligosaccharides, including the fragments of galactomannans main chain [2]. α-Galactosidases occur widely in microorganisms, plants and animals [3]. Microorganisms have the advantage of high production yields, and among them, fungal galactosidases are the most suitable for technological applications mainly due to their extracellular secretion, acidic pH optima, and broad stability profiles. Filamentous fungi have been extensively employed on an industrial scale for many decades in the production of a variety of enzymes [4].

α-Galactosidases have a number of biotechnological, medical and industrial applications. The most important industrial application is in the sugar-making industry [5] by the removal of raffinose from sugar beet molasses to facilitate the crystallization and improve the yield of sucrose.

The α-galactosidase gene of interest is located on chromosome 5 of *A. fumigatus* genome. During growth of the strain, *A. fumigatus* IMI 385708, on 2% locust bean gum (LBG), Puchart et al. [6] observed high levels of extracellular α-galactosidase production.

A. fumigatus is a saprophytic, thermotolerant, and haploid fungus. Among the 182 recognized species of *Aspergillus* [7], *A. fumigatus* is the most common human and animal pathogen. The opportunistic fungus mainly affects immunocompromised patients [8]. However, *A. fumigatus* is a good producer of many hydrolytic enzymes. The ability of *A. fumigatus* α-galactosidase to debranch polymeric legume seed galactomannans is quite rare among microbial α-galactosidases. This feature is common to α-galactosidases from *Penicillium simplicissimum* AGLI, *P. ochrochloron* and *A. niger* [9–11]. Degree of polymeric galactomannans degalactosylation by *A. fumigatus* enzyme was found to be the highest among microbial α-galactosidases and approached that by enzymes from plants [12,13].

A fairly new expression host from *A. sojae* ATCC strain was developed by Margreet Heerikhuisen, Cees van den Hondel and Peter Punt, TNO, The Netherlands. *A. sojae* ATCC11906 (*pyr*G$^-$) strain [14], having low proteolytic activity and being a uridine auxotrophic mutant which is unable to produce the enzyme orotidine-5-monophosphate decarboxylase, involved in the biosynthesis of uridine, was used for heterologous expression.

In the present study, α-galactosidase gene of the human pathogen *A. fumigatus* (*agl*B) was ligated onto the fungal expression vector, pAN52-4 (accession no. Z32699), and transformed into *A. sojae* ATCC11906 (*pyr*G$^-$) strain, a GRAS organism.

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2. Materials and methods

2.1. Strains, media, and cultivation

The thermotolerant filamentous fungus, *A. fumigatus* strain IMI 385708, which was formerly known as *Thermomyces lanuginosus* IMI 158749 (International Mycological Institute, Kew, Surrey, UK) [15] was cultivated on YpSs agar (4 g/L yeast extract, 1 g/L% K_2HPO_4, 0.5 g/L MgSO4·7H2O, 15 g/L starch, 2% agar) at 45 °C until sporulation and then stored at 4 °C. For induction of α-galactosidase gene, the culture was grown in YpSs broth using 0.5% LBG instead of starch at 45 °C and 155 rpm.

Escherichia coli XL1-Blue MRF was supplied by Stratagene (La Jolla, USA) and maintained according to the instructions of the supplier.

A. sojae ATCC11906 (*pyr*G$^-$) strain was kindly supplied by Dr. P.J. Punt (TNO Nutrition and Food Research, Dept. of Microbiology, The Netherlands). The stock cultures of *A. sojae* were grown on glucose containing modified YpSs agar with uridine and uracil supplementation. For the enzyme production, recombinant *A. sojae* was cultivated on modified YpSs broth (4 g/L yeast extract, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7\ H_2O$, and 20 g/L glucose) without uridine and uracil supplementation. The cultivations were carried out in 250 ml Erlenmeyer flasks with 100 ml working volume and incubated at 30 °C (in case of *A. fumigatus* at 45 °C) in a shaker incubator at 155 rpm.

2.2. Cloning and characterization of aglB gene

Based on the nucleotide sequence of *agl*B obtained from the *A. fumigatus* genome database of the Institute for Genomic Research website at http://www.tigr.org, two specific oligonucleotides with tails having *Hind*III cut sites were designed (5′-*GTATGCAGCC***AAGCTT**CTCGTCAGACCGGGCAA-3′ and 5′-*GTATGCAGC***AAAGCTT**TAGTCAGACCGGGCAATG-3′, where non-homologous tails are represented in italics) and used in PCR under the following conditions: denaturation at 94 °C, annealing at 55 °C and extension at 72 °C. The resulting PCR fragment was cloned onto vector pAN52-4 (Fig. 1). Insert was ligated onto pAN52-4 from the unique *Hind*III cut site (Gen Bank accession number Z32699). Ligation mixture composed of T4 DNA ligase, PCR fragment, ligation buffer and vector was incubated at 4 °C. Genomic DNA of *A. fumigatus* was isolated by QIAGEN's DNeasy plant mini kit. Plasmid DNA was purified with the standard alkaline lysis procedure [16].

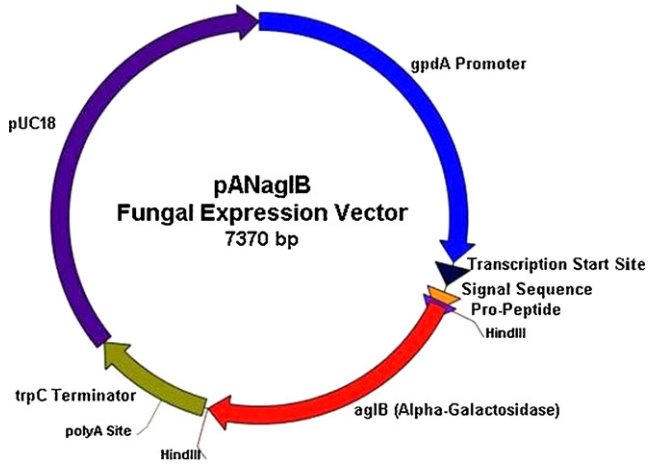

Fig. 1. Recombinant plasmid for the expression of *agl*B of *A. fumigatus*. Expression of the gene of interest was under the control of the *gpd*A promoter. Major transcription start site, poly A tail, *Hind*III cut site used to insert the gene, signal and pro sequences are illustrated on the drawing.

The insert was extracted from the agarose gel by Gel Extraction kit of Fermentas.

2.3. Fungal transformation and heterologous expression in A. sojae

The pAN52-4 expression vector was kindly provided by Dr. P.J. Punt. pAN52-4 is a fungal expression and secretion system, including *A. nidulans* glyceraldehydes-3-phoshate dehydrogenase (*gpdA*) promoter which is a strong and constitutive promoter and *trpC* terminator region and *A. niger* glucoamylase gene (*gla*A) preprosequence region [17,18]. On the expression vector, the nucleotide sequence of *agl*B signal sequence was replaced by the signal and prosequences of *A. niger gla*A gene and the gene was put under the control of the (*gpd*A) promoter. The final construct was named as pANaglB. In the cotransformation pAMDSPYG plasmid was used as a selective marker. This plasmid includes *amdS* gene of *A. nidulans* and *pyrG* gene from *A. niger*. The *amdS* gene encodes the enzyme that confers the ability to use acetamide and acrylamide as nitrogen and carbon sources and the *pyrG* gene encodes orotidine-5-monophosphate decarboxylase. The host strain *A. sojae* ATCC11906 (pyrG$^-$), which is a uridine auxotroph, was pre-grown in YpSs agar (see Section 2.1) with uridine and uracil supplementation at 30 °C and 155 rpm for 18 h and was transformed with the selective plasmid pAMDSPYG and pANaglB, at the same time, at a ratio of 1:10, as described by Punt and van den Hondel [19]. Transformants were selected for uridine prototrophy on YpSs medium without supplementation. Fungal transformants were cultivated at 30 °C and 155 rpm modified YpSs broth, containing 2% glucose instead of starch in 250 ml Erlenmeyer flasks with 100 ml working volume.

2.4. Assay of α-galactosidase activity

0.25 ml of appropriately diluted culture supernatant from the third day of the cultivation and 0.25 ml of 4 mM *para*-nitrophenyl-α-D-galactopyranoside (*p*NPG) were mixed to a final volume of 1 ml in 100 mM phosphate buffer (pH 4.5) and incubated at 50 °C for 8 min. As the blank solution; 0.25 ml of 4 mM *p*NPG was mixed with 100 mM phosphate buffer (pH 4.5) to a final volume of 1 ml. The reactions were terminated by the addition of 3.5 ml 0.2 M sodium tetraborate solution. The *p*-nitrophenol released was measured from the absorbance at 410 nm using Shimadzu UV–visible spectrophotometer, 1700 PharmaSpec and *p*-nitrophenol standard curve. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute under the conditions specified above. The data presented for all α-galactosidase activity determinations were mean values of duplicate assays, in which the standard deviations always lay under 10%.

2.5. Analysis of protein profiles

Extracellular proteins were analyzed by SDS-PAGE according to Laemmli [20]. Culture supernatants of the third day cultivation were collected by filtration through Whatman no. 1 filter paper and concentrated 2-fold in vacuum evaporator. Electrophoresis system, Serva Blue Flash S, 15 cm × 28 cm × 8.5 cm was used and protein bands were stained with 0.2% (w/v) Silver Nitrate solution.

3. Results

According to the derived amino acid sequence of *A. fumigatus*, *agl*B gene encodes an extracellular protein of 447 amino acids with a 22 amino acids long signal peptide. The plasmid DNA of the construct was sequenced and aligned with the published sequence of *A. fumigatus* genomic DNA (data not shown). As described in

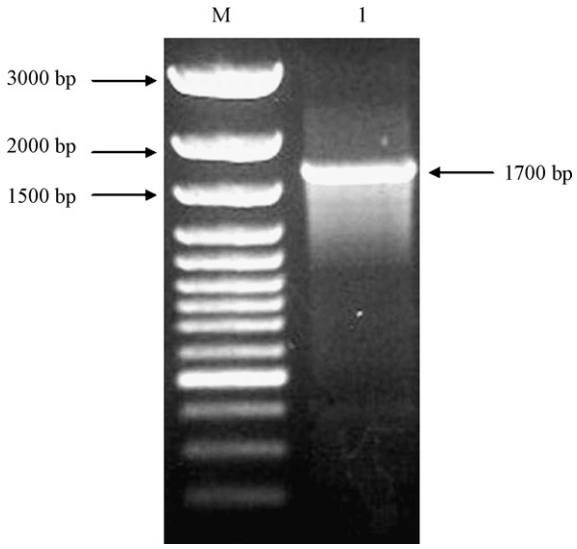

Fig. 2. Isolated 1700 bp PCR fragment amplified by specific primers from *A. fumigatus* genomic DNA (lane 1), GeneRuler 100 bp DNA Ladder Plus (lane M).

Section 2.2, the mature protein coding region of *agl*B was cloned by PCR followed by a ligation on vector pAN52-4. On the construct after signal peptide cleavage 5 extra amino acids (Ala, Gly, Ser, Glu, Ala) were introduced to the N-terminus of the mature protein. This insertion was inevitable due to the nature of the ligation process. The PCR fragment amplified by specific primers yielded 1700 bp as shown in Fig. 2. Following *Hind*III digestion, two expected bands [insert, *agl*B (1622 bp) and vector, pAN52-4 (5748 bp)] were analyzed by agarose gel electrophoresis as shown in Fig. 3. The cloned gene (*agl*B) was sequenced with gene bank accession number (FJ843023).

In the first step of cotransformation, *A. sojae* protoplasts were formed and transformed with a mixture of recombinant plasmid pAN*agl*B and selective plasmid pAMDSPYRG as described in Section 2.3. To confirm the presence of *agl*B gene in the recombinant transformants α-galactosidase activity screening was performed.

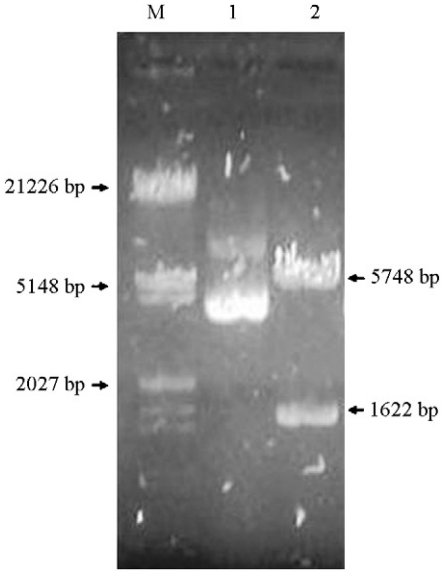

Fig. 3. Restriction digestion of recombinant plasmid with *Hind* III. Isolated recombinant plasmid (lane 1), *Hind* III-cut recombinant plasmid (lane2), Lambda DNA/*Eco*RI + *Hind*III marker (lane M).

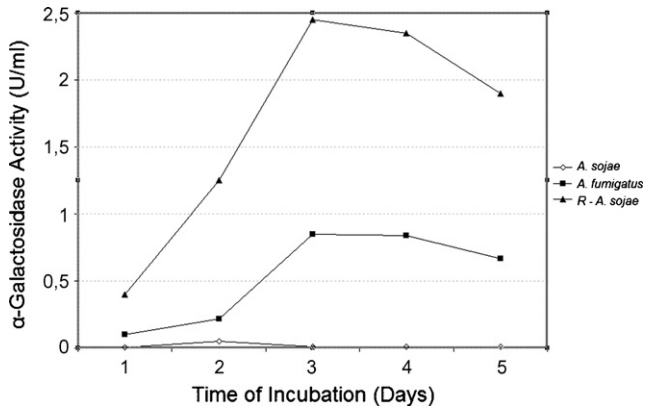

Fig. 4. Comparison of the extracellular α-galactosidase activities of control strain *A. sojae*, *A. fumigatus* and recombinant *A. sojae* (R-*A. sojae*).

To induce α-galactosidase production *A. fumigatus* strain was grown in the LBG containing medium. Since α-galactosidase gene was inserted into the integrative expression vector pAN52-4 bearing the constitutive and strong *gpd*A promoter, induction of α-galactosidase gene in recombinant *A. sojae* was not required and expression was possible on simple carbon sources like glucose. Fig. 4 is the time course graph showing the comparison of extracellular α-galactosidase activities of the control strain *A. sojae*, recombinant *A. sojae* (*A. sojae* Ta1) and *A. fumigatus*.

The production of α-galactosidase in recombinant *A. sojae, A. fumigatus,* and non-recombinant *A. sojae* strain were compared after 3 days of incubation. 2.45 U/ml production was observed in recombinant *A. sojae* strain which was nearly 3-fold higher than in *A. fumigatus* (0.85 U/ml). In non-recombinant *A. sojae* strain, α-galactosidase activity was not observed (Fig. 4).

Extracellular protein productions of untransformed control strain *A. sojae* grown on glucose, recombinant *A. sojae* (R-*A. sojae*) grown on glucose and *A. fumigatus* grown on LBG containing media were compared. Results, shown in Fig. 5, were consistent with the results obtained from the activity measurement. While a distinct α-galactosidase protein band was observed in transformant *A. sojae*, no protein band around this position was observed in non-recombinant *A. sojae*. Due to induction with LBG, in addition to α-galactosidase, many protein bands probably belonging to other hydrolytic enzymes were observed in *A. fumigatus*. In addition,

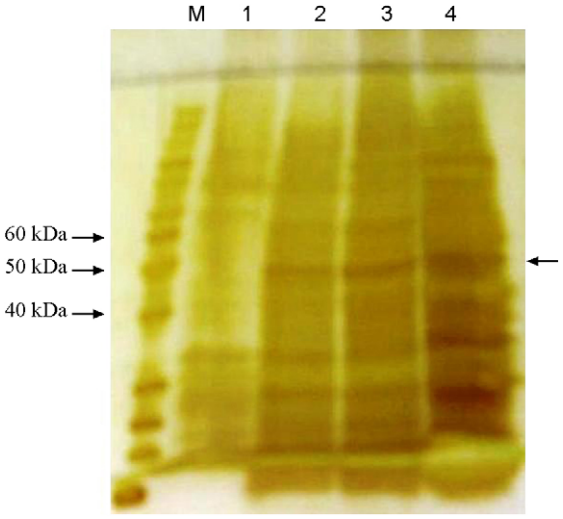

Fig. 5. SDS-PAGE's of culture supernatants. Lane 1, *A. sojae*; lanes 2 and 3, R-*A. sojae*; lane 4, *A. fumigatus*; lane M, protein molecular weight marker.

from the SDS-PAGE results, the molecular mass of *A. fumigatus* α-galactosidase was approximately 50 kDa, which was close to the expected value previously reported by Puchart [2].

4. Discussion

The expression system used in this study was very efficient and allowed us to obtain high heterologous expression of α-galactosidase in recombinant *A. sojae* in the absence of complex polymeric substrate. The use of strong promoters derived from housekeeping genes, such as the fungal glyceraldehyde-3-phosphate dehydrogenase gene, has the advantage of constitutive, growth-associated production of heterologous proteins. The 5 extra amino acids on the N-terminus of the transformant mature protein arises from the way of primer design, none gives rise to the elevation of expression levels. Since the PCR amplification was done by a proofreading enzyme from the template DNA, there were only a few silent mutations. Instead of the 5 amino acids inserted extra, no other changes occurred in the amino acid sequence. In *A. fumigatus*, α-galactosidase production requires induction and the best production is on LBG. However, LBG makes the medium viscous and *A. fumigatus* produces many other hydrolytic enzymes to degrade LBG, resulting in a mixture of enzymes, perhaps also including other α-galactosidases. This is a disadvantage for the purification process and indicates that the observed α-galactosidase activity from the *A. fumigatus* culture supernatants is the total effect of different α-galactosidases. In the genome of *A. fumigatus* there are 6 putative α-galactosidase genes [21]. Thus, the observed 3-fold increases in α-galactosidase production by *A. sojae* is likely to be higher than the observed level.

Further studies are now in progress to study the effects of different osmotic stresses on heterologous production system using *gpd*A promoter, to optimize the culture conditions for α-galactosidase production in recombinant *A. sojae* and to characterize the recombinant enzyme. Expression system used in this study can also be used for researching into the mechanism of transgalactosylation, applying site-directed mutagenesis, analyzing the effect of upstream control regions of the gene on expression and secretion or manufacturing any product on industrial scale.

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